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Modulation of mammalian cell proliferation by a modified tRNA base of bacterial origin

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Addition of the q-base to q-deficient non-transformed mammalian cells stimulated their proliferation. The q-base also improved proliferation of some cancer-derived cell lines, but inhibited proliferation of others. The proliferation of HeLa-S3 carcinoma cells was stimulated by q under aerobic conditions, but was inhibited when the cells had shifted their energy metabolism towards glycolysis as the result of oxygen limitation. Q-deficient cells could not adapt their proliferation to the respective oxygen tension. The q-base stimulated the proliferation of non-transformed fibroblasts but inhibited proliferation of the same cell line, when aerobic glycolysis was increased after transformation with the *ras* gene. The results suggest that the q-base permits mammalian cells to adapt their proliferation to their specific metabolic state.

Queuine; Cell proliferation; Hypoxia; Oncogenic transformation; tRNA modification; Aerobic glycolysis

1. INTRODUCTION

More than 50 different modified nucleosides have been characterized in tRNA [1], yet their functional role is not clear. Many of these modifications are not essential for tRNA function but may be involved in regulation. The highly modified tRNA nucleoside, queuosine (Q), replaces guanosine (G) in position 34 of the anticodon of bacterial and eukaryotic $tRNAs_{GUN}$ [2–4]. Queuosine is synthesized by eubacteria, involving unique *S*-adenosylmethionine- and vitamin B12-dependent steps [5–8]. The occurrence of Q-containing tRNAs is highly conserved throughout the animal kingdom [2,3]. However, mammals cannot synthesize Q but are supplied with the free q-base (queuine, in analogy to guanine)-derived from bacterial tRNA- by nutrition and by their intestinal flora [9]. The eukaryotic tRNA:guanine transglycosylase (EC 2.4.2.29) replaces guanine (g) with queuine (q) in the corresponding tRNAs in a base for base exchange reaction without breakeage of the sugar phosphate backbone [9]. Thus, q is present in mammals as a free base outside and inside the cell, and in a tRNA-bound state within the cell.

Hypomodification of Q-tRNAs is frequently observed in neoplastically transformed cells and has been linked with the proliferative capability and/or malignancy of such cells [10–13]. Intraperitonial injection of q into ascites tumor bearing mice relieves Q-deficiency of tRNAs, correlating with inhibition of tumor growth [14]. Characteristic fluctuations in the amount of hypomodified Q-tRNAs are associated with the transition from a proliferative stage to differentiation [2]. The 'Q-system' – comprising the free q-base and its tRNA-bound state, queuosine – participates in control mechanisms gouverning oxidative and glycolytic metabolism [15–18]. Q-modification of tRNAs was proposed to be related to changes in saturation density in primary skin fibroblasts. [19]. HeLa cells grown in medium supplemented with q-free horse serum [3] do not contain Q in tRNA (q-deficient cells). Addition of chemically synthesized q to these cells modulates their proliferation depending on oxygen availability [20]. It was proposed that q interacts with regulatory proteins responsible for growth control, as deduced from changes in the expression of the growth-associated genes c-*fos* and c-*myc* [21], and in the tyrosine phosphorylation of specific membrane-associated proteins [22]. In addition, the q-base modulates the autophosphorylation activity of the receptor for epidermal growth factor (EGF) in vitro and in intact cells and inhibits EGF-supported proliferation of HeLa cells [23]. A possible involvement of queuine/Q-tRNAs in mechanisms of growth control has been proposed also by French et al. [24], who described that q prevents differentiation and favours proliferation of HL-60 cells.

Here we have investigated the effect of q on the proliferation of non-transformed cell lines, tumor-derived cell lines, and cells transformed by the activation of discrete oncogenes. The results suggest that q is a modulator of the proliferation of mammalian cells in culture. Queuine can act as a growth-stimulator or as a

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growth-inhibitor, depending on the aerobic or glycolytic state of cells.

2. MATERIALS AND METHODS

Minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, and dialyzed fetal calf serum (dFCS) were from Gibco/BRL (Germany, Japan). Queuine-free adult horse serum [3] was from Boehringer Mannheim (Germany). [8-^{3}H]guaninesulfate was synthesized by Amersham (Germany). The q-base was synthesized by Drs. H. Akimoto and H. Nomura of the Central Research Laboratories, Takeda Chemical Ind. Ltd., Osaka, Japan [25].

Mammalian cells were cultivated aerobically in an atmosphere of 95% air and 5% CO_2, and under hypoxic conditions in an incubator from Labotect, Germany, in which the atmosphere was replaced by nitrogen to an oxygen content of 7%. Non-transformed, established cell lines: human epithelial-like hepatocytes (Chang Liver) and embryonic lung fibroblasts (Wi-38); mouse embryonic fibroblasts (NIH-3T3). Cancer-derived cell lines: human epithelial-like cervical carcinoma cells (HeLa-S3), lymphoblast-like promyelocytic leukemia cells (HL-60), epithelial-like vulval carcinoma cells (A-431), and colon adenocarcinoma cells (Colo-DM320); rat adrenal pheochromocytoma cells (PC-12); mouse Ehrlich Lettre Ascites tumor cells (EAT). The cell lines Chang Liver, PC-12, and Wi-38 were purchased from the American Type Culture Collection (ATCC). The cell lines HeLa-S3, EAT, and HL-60 were provided by Dr. A. Ogilvie, and Colo-DM320 by Dr. T. Dingermann (Erlangen, Germany). A-431 cells were provided by Dr. K. Yamane (National Cancer Center Research Institute, Tokyo, Japan); non-transformed NIH-3T3 fibroblasts and NIH-3T3 cells transformed by the activated c-*Ha-ras* gene [26] were provided by Dr. T. Sakiya, c-*Ki-ras*-transformed NIH-3T3 cells by Dr. Y. Taya, *N-ras*-transformed NIH-3T3 cells by Dr. M. Terada, and *raf*-transformed NIH-3T3 cells [27] by Dr. M. Nagao (National Cancer Center Research Institute, Tokyo, Japan). NIH-3T3 cells transformed with the *erbB-2* gene [28] were provided by Dr. T. Yamamoto (Institute of Medical Sciences, University of Tokyo, Japan). Cells were grown and maintained in MEM supplemented with 10% q-free horse serum or dialyzed fetal calf serum. RPMI 1640 medium was used in the case of HL-60 cells. A-431 cells were cultivated in DMEM/10% dFCS, and NIH-3T3 cells and the respective *ras*-, *erbB-2*-, and *raf*-transformed cells in DMEM/5% dFCS. Cells were seeded to 10 cm culture dishes or 24-well culture vessels at low density and precultivated for 2 or 3 days without queuine. The medium was then replaced with fresh medium, and the cells were further incubated either with or without q (final concentration 300 nM). Wi-38 cells and Colo-DM320 cells were supplied with q without exchange of the medium. HL-60 cells and EAT-cells were seeded directly into q-containing or q-free medium without precultivation. Adherent growing cells were detached by trypsinization and collected by centrifugation. The cell number was determined either microscopically by counting in a Fuchs/Rosenthal chamber, or automatically with a coulter counter (CASY 1, Schärfe System, Germany). For the statistical evaluation of the data from repeated experiments, the increase in the cell number of (+q)-cultures after addition of q was determined at the point of maximal response to q, and is expressed as fold increase in the cell number of (−q)-cultures (relative increase Δ+q/Δ−q) within the same period.

For the determination of total specific LDH activity, cells were grown without q for 3 days under aerobic or hypoxic conditions. The culture medium was exchanged and the cells were harvested 24 h later. Total LDH activity in cytosolic extracts was measured spectrophotometrically according to Schwarz and Bodansky [29].

For the isolation of tRNA, cells ($1-5 \times 10^8$ for each analysis) were collected by scraping and centrifugation, washed with PBS, frozen in liquid nitrogen, and stored until use at −20°C. The frozen cell pellet was overlayed with 2 ml acetate buffer (140 mM Na-acetate, pH 4.5) and 2 ml phenol. The sample was mixed vigorously for 1 h at room temperature. The aqueous phase was collected, extracted once with chloroform/isoamylalcohol (24:1), and loaded onto a DEAE-cellulose column (Whatman DE52, 1 ml volume) equilibrated with acetate buffer. The column was washed with 0.35 M NaCl in acetate buffer, and the tRNA was eluted with 0.55 M NaCl. Nucleic acid was precipitated with 0.8 vol of isopropanol, collected by centrifugation at 10,000 g, washed with 70% ethanol, dried under vacuum, and re-dessolved in 1 ml 10 mM Tris/HCl, pH 8.0. The extent of tRNA modification with Q was determined as described by Okada et al. [10] using the *E. coli* tRNA:guanine transglycosylase. This enzyme catalyzes the exchange of tRNA-bound guanine for radioactive guanine specifically at position 34 of the tRNAs of the Q-family, but cannot exchange tRNA-bound queuine for guanine. Therefore, the guanine acceptance of tRNA is a measure for the extent of Q-modification.

3. RESULTS

3.1. *Queuine can act as promoter or as inhibitor of mammalian cell proliferation*

The effect of q on the proliferation of established non-transformed cell lines and cancer-derived cells was investigated. Cells were grown and maintained in q-free medium and therefore did not contain Q in tRNA. The addition of q to q-deficient non-transformed human and mouse cells (Chang Liver, NIH-3T3, and Wi-38) stimulated their proliferation (Fig. 1A). The response of cancer-derived, q-deficient cells to the addition of q can be divided into two categories (Fig. 1B): (i) proliferation was stimulated in the case of the human cell lines HeLa-S3, A-431, and HL-60; (ii) proliferation was inhibited after addition of q in the case of Colo-DM320 cells, PC-12 cells, and Ascites tumor cells (EAT). The results show that q can act as a positive or a negative modulator of the proliferation of cultured mammalian cells. Growth-modulation by q in the case of cancer-derived cells apparently is cell-type specific, suggesting that a certain type of predisposition of the cell determines whether q stimulates or inhibits proliferation.

3.2. *Modulation of proliferation by q is related to the activation of discrete oncogenes*

Inhibition of proliferation by q was observable in cancer-derived cells but not in non-transformed cells. We therefore reasoned that growth inhibition might be related to the activation pattern of cellular oncogenes. Therefore, the effect of q-addition on proliferation was compared between q-deficient non-transformed NIH-3T3 cells and the same cell line that was transformed with the activated oncogenes c-*Ha-ras*, c-*Ki-ras*, *N-ras*, c-*erbB-2* and c-*raf*, respectively. NIH-3T3 cells showed a maximal response already 24 h after addition of q (Fig. 1). We therefore chose this point (comprising approximately one population doubling) to measure differences in the proliferation of the respective cell lines (Table I). Addition of q to q-deficient NIH-3T3 cells stimulated their proliferation within 24 h of further incubation under aerobic conditions. However, proliferation of NIH-3T3 cells transformed with the activated oncogenes c-*Ha-ras*, *N-ras*, c-*Ki-ras*, and c-*erbB-2* was inhibited after addition of q. In contrast, NIH-3T3 cells

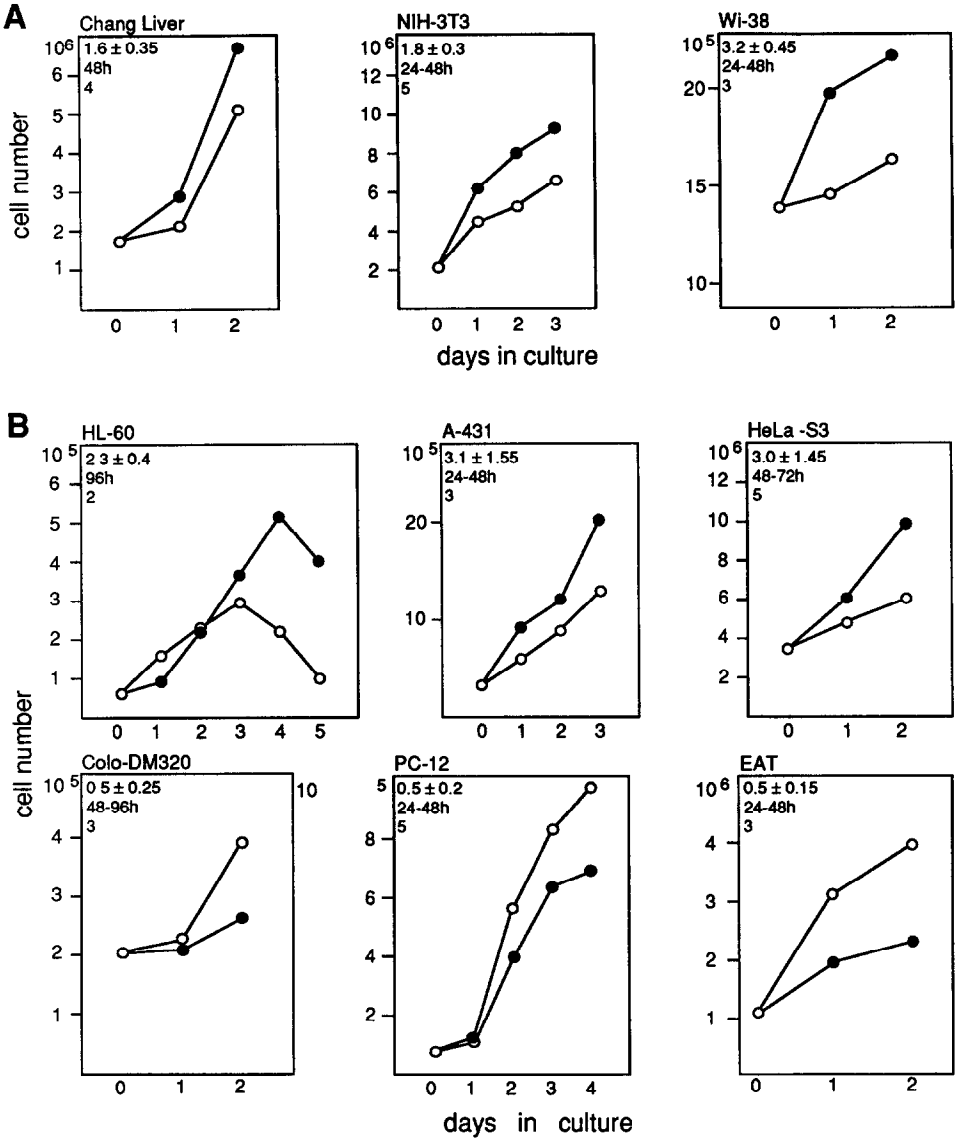

Fig. 1. Proliferation of non-transformed (A) and cancer-derived mammalian cells (B) in the absence (-○-) or presence (-●-) of q. The cells were precultivated in the absence of q. At $t = 0$, half of the cultures were supplied with 300 nM q, the second half received no q. The total cell number per culture vessel was determined at the indicated times after addition of q. Numbers within blot frames show in the following order: the mean value of the relative increase (Δ+q/Δ−q) in cell number of +q-cultures ± variation found in repeatet experiments (see method section), the time after q-addition when these values were obtained, and the number of experiments performed with the respective cell line.

transformed with the activated c-*raf* gene, responded only very little to supplementation with q; at least, no inhibition of proliferation was observed after addition of q to these cells. The results indicate that positive or negative modulation of the proliferation of transformed cells by q might depend on the activation of discrete oncogenes.

3.3. *Modulation of proliferation by q is related to the metabolic state*

Q-deficient HeLa cells grew equally well under aerobic and under hypoxic conditions. (Fig. 2). In the presence of q, proliferation of HeLa cells was improved under aerobic but reduced under hypoxic conditions, indicating that the q-base is essential for this kind of growth-regulation. Based on these results we assumed that growth-modulation by q might depend on whether the cells have a more aerobic or a more glycolytic metabolic predisposition. We therefore investigated metabolic changes caused by hypoxia. Under reduced oxygen tension, HeLa cells activate transcription of the *ldh a* gene, coding for lactate dehydrogenase A (LDH A), the terminal enzyme of anaerobic glycolysis in mammals [30]. This induced gene expression is paralleled by an increase in the amount of LDH A protein, and also an increased activity of the muscle type isoenzyme LDH 5, the homotetrameric form of LDH A (LDH A4). Specific LDH activity was about twice as high in hypoxi-

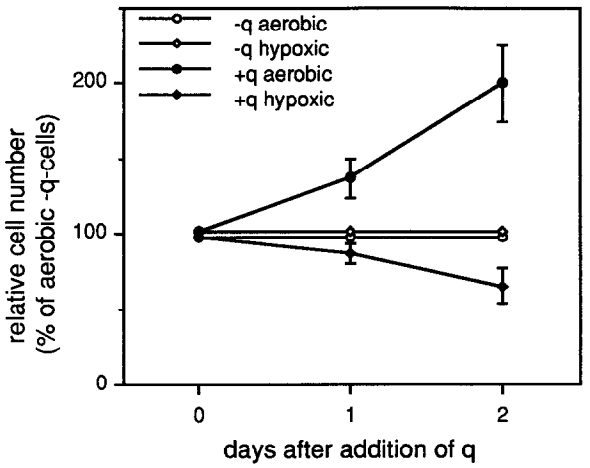

Fig. 2. Proliferation of q-deficient (−q) and q-supplemented (+q) HeLa cells under aerobic and hypoxic conditions. The relative increase in cell number is expressed as percentage of the cell number of aerobically grown q-deficient cells. Values are means from five independent experiments.

cally than in aerobically growing, q-deficient HeLa cells (Fig. 3), suggesting that hypoxically growing cells had shifted their energy metabolism towards glycolysis. Oncogen activation has been shown to increase glycolysis and LDH activity in rat cells under aerobic conditions [31,32]. Specific LDH activity was two-fold higher in *ras*-transformed NIH-3T3 cells than in non-transformed cells (Fig. 3), suggesting that aerobic glycolysis is increased also in mouse fibroblasts after activation of the *ras* gene. This was confirmed by the observation that *ras*-transformed cells also showed a three-fold higher rate of lactate production than the non-transformed cells (not shown). The results indicate that modulation of cell proliferation by q is related to the switch from an oxygenated to a more glycolytic state of the cell.

3.4. *Modification of tRNAs with q*

The extent of modification of tRNAs with q was proposed to be related to the metabolic state of cells and tissues [3]. A relationship between the extent of modification of Q-tRNAs and the proliferative capability of tumor cells has been proposed [11–13,24]. We investigated the extent of Q-modification of tRNAs in representative cell lines 24 h after addition of q to q-deficient cells. Although incomplete, the extent of tRNA modification with q was the same in aerobically and in hypoxically grown serum-supplemented HeLa cells (Table II). However, we observed that q cannot be inserted into tRNA in hypoxically grown HeLa cells under conditions when serum factors (growth factors) become limiting (unpublished results). The tRNAs were completely modified with q in NIH-3T3 fibroblasts and in the corresponding *Ha-ras*-transformed fibroblasts. Addition of q to A-431, HL-60, Colo-DM320, and EAT cells also relieved Q-deficiency of tRNAs in all cases (Table II).

Table I

Differential modulation of the proliferation of normal and transformed fibroblasts by q

Cell line	Relative increase in cell number (Δ+q/Δ−q)[a]
NIH-3T3 non-transformed	1.8 ± 0.3
Ki-ras-transformed NIH-3T3	0.5 ± 0.1
Ha-ras-transformed NIH-3T3	0.5 ± 0.2
N-ras-transformed NIH-3T3	0.5 ± 0.1
erb-2-transformed NIH-3T3	0.5 ± 0.3
raf-transformed NIH-3T3	1.2 ± 0.3

[a] The increase in cell number of (+q)-cultures was determined 24 h after addition of q, and is expressed as fold increase in the cell number of (−q)-cultures within the same period.

4. DISCUSSION

The growth-regulating function of isopentenyladenine and derivatives (cytokinins) in plants has been well established [33]. These adenine derivatives are also constituents of tRNA. Cytokinins can act as inhibitors or as promoters of proliferation, depending on the developmental stage of cells and on the concentration used. They also affect protein synthesis, enzyme activities and organellar function. Here we show that the modified guanine analogue, queuine, a nutrition factor for mammals, functions as a growth regulator in mammalian cells. Like the cytokinins in plants, q also occurs as a free base and as modified nucleoside in tRNA. The growth-modulating activity of q probably is of general significance for cultured mammalian cells, because human, mouse and rat cells of different origin and morphology were responsive to q-addition. Like cytokinins, q also affects cellular parameters such as protein synthesis [18], organellar function (mitochondria), and the activity of enzymes such as lactate dehydrogenase and superoxide dismutase [30,17]. The responses of mammalian cells to treatment with q appear to be very similar to the responses of plant cells to treatment with cytokinines, suggesting that q may have functions in mammals that are comparable with the function of cytokinins in plants.

Queuine can act as a positive or a negative modulator of cell proliferation. What determines whether q inhibits or stimulates proliferation? It is a long known phenomenon first described by Warburg [34] that glycolysis is increased in tumor cells even under aerobic conditions (aerobic glycolysis). This has been shown for EAT cells used in this study [35]. Racker et al. described that transformation of rat cells with the activated *ras* gene – but not the *myc* gene – increases aerobic glycolysis [31], suggesting that activation of some – but not all – oncogenes may cause a shift of the metabolism towards

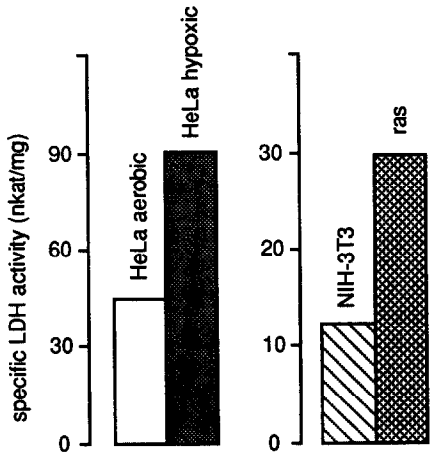

Fig. 3. Specific LDH activity in aerobically and hypoxically grown HeLa cells, and in non-transformed and *ras*-transformed NIH-3T3 fibroblasts. Cells were grown without q for 2 days. The culture medium was exchanged and cells were harvested 24 h later. Total specific LDH activity was measured in cytosolic extracts.

glycolysis under aerobic conditions. Glycolysis is increased also after exposure of cells to hypoxic conditions as in the case of HeLa cells. Apparently, q improves proliferation of cultured mammalian cells under conditions that favour respiration, whereas an inhibitory effect of q on proliferation is associated with a shift of the energy metabolism towards glycolysis either as a consequence of oxygen-limitation, or after transformation with those oncogenes that cause an increase in aerobic glycolysis. Probably, the Q-system provides a means for mammalian cells to regulate proliferation in dependence on the metabolic state, and q-deficiency results in a loss of this ability, as is the case in HeLa cells.

Hypomodification of tRNAs of the Q-family is frequently observed in transformed cells, and has been proposed to have some regulatory function. Some observations suggested a relation between the extent of Q-modification of tRNAs and the malignancy and proliferative capability of tumor cells [11,13]. However, Q-deficient tRNAs can also be extracted from fast proliferating undifferentiated cells and regenerating liver [2], suggesting that this feature does not exclusively apply to cancer cells. Here we showed that addition of q to cells that contained only G-tRNAs (q-deficient cells) resulted in the appearence of Q in tRNA in any case. However, the proliferation of some cell lines was stimulated whereas that of others was inhibited after q-addition. These results suggest that growth-modulation by q is not directly related to the absence or presence of Q-containing tRNAs, but depends on the aerobic or glycolytic state of cells and on the availability of the free q-base.

Table II

Q-modification of tRNAs 24 h after addition of q to q-deficient cells

Cell line	% Modified tRNAs[a]
HeLa aerobic	68
HeLa hypoxic	70
NIH-3T3	100
Ha-ras transformed NIH-3T3	100
A-431	100
Colo-320DM	56
HL-60	100
EAT	100

[a] In this test, the guanine acceptance of bulk tRNA is determined. Cells grown without q contain only Q-deficient tRNAs and the guanine acceptance is 100%, equivalent to 0% Q-modification. No guanine acceptance means 100% Q-modification of tRNAs.

Treatment of HeLa cells with q reduces the in vitro phosphorylation of a membrane associated protein that becomes phosphorylated at tyrosine residues after stimulation with the epidermal growth factor. Reduced tyrosine phosphorylation of this protein in the presence of the q-base is observed also in an in vitro system devoid of tRNAs [22]. Furthermore, simultaneous stimulation of q-deficient intact HeLa cells and A-431 cells with EGF and q results in an increased autophosphorylation activity of the EGF receptor tyrosine kinase, compared with stimulation with EGF in the absence of q [23]. Stimulation of autophosphorylation is also observed in vitro using either a tRNA-free membrane preparation, or the immuno-purified EGF receptor. Treatment of q-deficient HeLa cells with low doses of EGF stimulates proliferation. This EGF-mediated increase in proliferation is completely prevented in the presence of q [23]. These data suggest that the free q-base modulates mitogenic signaling pathways by influencing tyrosine phosphorylation of specific proteins involved in growth control. Probably, inhibition of proliferation by q, as was observed in transformed cells, is achieved through inhibition of the EGF receptor system that is known to be present in most mammalian cells, whereas stimulation of proliferation is based on a different mechanism that may involve Q-modification of tRNAs as proposed by French et al. [24]. The Q-system may be of special significance in embryogenesis and in tumorigenesis, where a shift from a respiratory to a glycolytic state can be observed [36], and where q was found to accumulate [3]. The high degree of evolutionary conservation of this system throughout the animal kingdom supports this assumption.

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